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14. ABSTRACT

Mitogen-activated protein kinase kinase 4 (MKK4) is a dual-specificity kinase with a critical role in regulating the activity of the c-Jun-N-terminal kinase (JNK) and p38 kinases. We identified a novel biological function for MKK4 in the regulation of growth of ovarian and prostate cancer metastases. Previous clinical correlative studies showed that MKK4 protein levels were reduced in high-grade prostate cancer and prostate and ovarian cancer metastases as compared to normal tissue controls. These findings prompted the investigation of mechanism(s) responsible for down-regulation of MKK4 in a panel of cancer cell lines. Initial studies found that low levels of MKK4 protein did not correlate with either exon deletion or decreased levels of MKK4 mRNA, suggesting that MKK4 protein levels were regulated by either reduced translation or reduced protein stability. The endogenous MKK4 protein was very stable and did not appear to be subject to altered proteolysis. Instead, MKK4 biosynthesis appeared may be regulated by altered translation. In support of this assertion, we found that the cytosolic MKK4 mRNA was shifted towards active polysomes in cells with higher levels of MKK4 protein, suggesting that MKK4 mRNA was translated more efficiently in these cells. This study supports a novel mechanism for the regulation of MKK4 protein levels. Further, these findings have potential therapeutic implications for modulating the expression of an important signaling kinase involved in the regulation of metastatic growth.

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INTRODUCTION: We have recently identified a novel function for the mitogen-activated protein kinase kinase 4 (MKK4) in metastasis suppression. MKK4 is a dual-specificity kinase with an important role in regulating the activity of the c-Jun N-Terminal Kinase (JNK) and p38 kinase. Ectopic expression of MKK4 in highly metastatic prostate cancer cells reduces the number of overt metastases by ~ 90% compared to controls, without affecting the growth rate of the primary tumors. Clinical studies have shown that MKK4 expression varies inversely with Gleason grade; however the gene is infrequently mutated in prostate cancer metastases. Similarly, the MKK4 protein is frequently downregulated, but the gene is not mutated, in prostate cancer cell lines derived from metastases. Taken together these findings prompt the question, "How is the MKK4 protein down-regulated in these cancer cells?" Although MKK4's kinase activity has been studied extensively, we were unable to locate any studies that examined the regulation of MKK4 protein expression. We anticipated that cells displaying undetectable MKK4 protein expression would have similarly low levels of MKK4 mRNA. To our surprise, we detected MKK4 mRNA in prostate cancer cell lines that lack MKK4 protein expression. This suggests that MKK4 protein levels are regulated by a post-transcriptional mechanism. Work supported by this DOD Hypothesis Development Award showed that low levels of MKK4 protein did not correlate with either exon deletion or decreased levels of MKK4 mRNA, suggesting that MKK4 protein levels were regulated by either reduced translation or reduced protein stability. The MKK4 protein was very stable and did not seem to be regulated by altered proteolysis. Instead, MKK4 biosynthesis appeared to be regulated by altered translation. In support of this notion, we show that the cytosolic MKK4 mRNA is shifted towards active polysomes in cells with high levels of MKK4 protein, suggesting that MKK4 mRNA is translated more efficiently in these cells. This study supports a novel mechanism for the regulation of MKK4 protein levels. Furthermore, these findings have therapeutic implications for modulating the expression of an important signaling kinase involved in the regulation of metastatic growth.

BODY: We have reported our findings for each of the experiments described in our Statement of Work. These results are also presented in detail with additional studies in the manuscript which is provided in the Appendix.

Experiment A: Is the MKK4 mRNA transcript sequestered in the nucleus? A critical, though often overlooked, step in gene expression is the transport of mRNA from the nucleus to the cytoplasm where it is translated into protein. Most cellular mRNAs appear to be exported at constant rates, but mRNA transport has been shown to critically dictate the expression patterns for some genes. To test the possibility that MKK4 mRNA is inefficiently transported from the nucleus in low MKK4-expressing cells, PC3 and LNCaP cells were fractionated into cytosolic and nuclear components. Northern blotting of RNA from cellular fractions demonstrated that MKK4 mRNA is primarily nuclear in both cell lines. Representative data are shown in Figure 1. Protein was also isolated from fractions to confirm the clean and specific preparation of cytoplasmic and nuclear lysates, and cytoplamic and nuclear markers (α-Tubulin and Lamin A/C, respectively) were tested. The majority of MKK4 mRNA is nuclear in both LNCap and PC3 cells, although no measurable difference in localization could be seen between the cell lines to explain the disparity in protein expression.

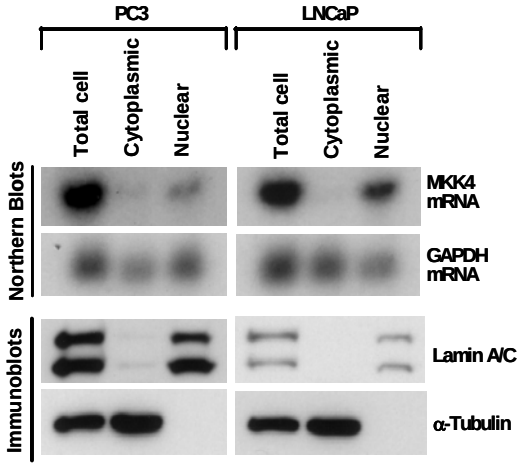


Fig 1. Localization of MKK4 in PC3 and LNCaP cells. Cells were separated into nuclear and cytosolic fractions, and RNA and protein were prepared. RNA was subjected to Northern blotting for MKK4 (top panels) and GAPDH was used as a loading control. Protein lysates were immunolotted for the cytoplasmic marker α-Tubulin and the nuclear marker Lamin A/C to confirm successful fractionation, and actin served as a loading control.

Experiment B: Is the MKK4 mRNA transcript failing to complete translation initiation? Having systematically excluded the involvement of several key regulatory mechanisms in determining MKK4 abundance, we asked whether the relative translation rate of MKK4 mRNA may underlie the observed differences in MKK4 protein levels. To this end, we studied the association of the MKK4 mRNA with the translational machinery in cells expressing different levels of MKK4. Although there are many levels of control within the process of translation, translational initiation is the rate-limiting step. The association of a given mRNA with polysomes of particular lengths reveals the degree to which that mRNA has completed translation initiation; consequently, this analysis provides a measure of the synthesis rate of the corresponding protein (Figure 2, A *left*). To ascertain the translational status of the MKK4 mRNA, we investigated its relative association with the translational apparatus. The cellular cytoplasmic components were size-fractionated using sucrose gradients (Figure 2, A, *right*). Following the extraction of total RNA from each fraction, the relative levels of the MKK4 mRNA (as well as those of GAPDH and β-Actin, encoding two housekeeping genes) were examined by Northern blot analysis. In a comparison of lysates from low-expressing (PC3 and DuPro) and high-expressing (LNCaP and C4-2) cells, the MKK4 mRNA in LNCap and C4-2 cells was found to be preferentially associated with polysomes of higher molecular weight, indicating a greater engagement of the MKK4 mRNA in translation in these cells. Representative data from PC3 and LNCaP cells is shown in Figure 2, B. This shift is nicely illustrated by graphing percent Northern signal in each pooled fraction from the sucrose gradient (Figure 2, C). Together, these observations point to the critical involvement of translational regulation as a key process governing the modulation of MKK4 protein levels.

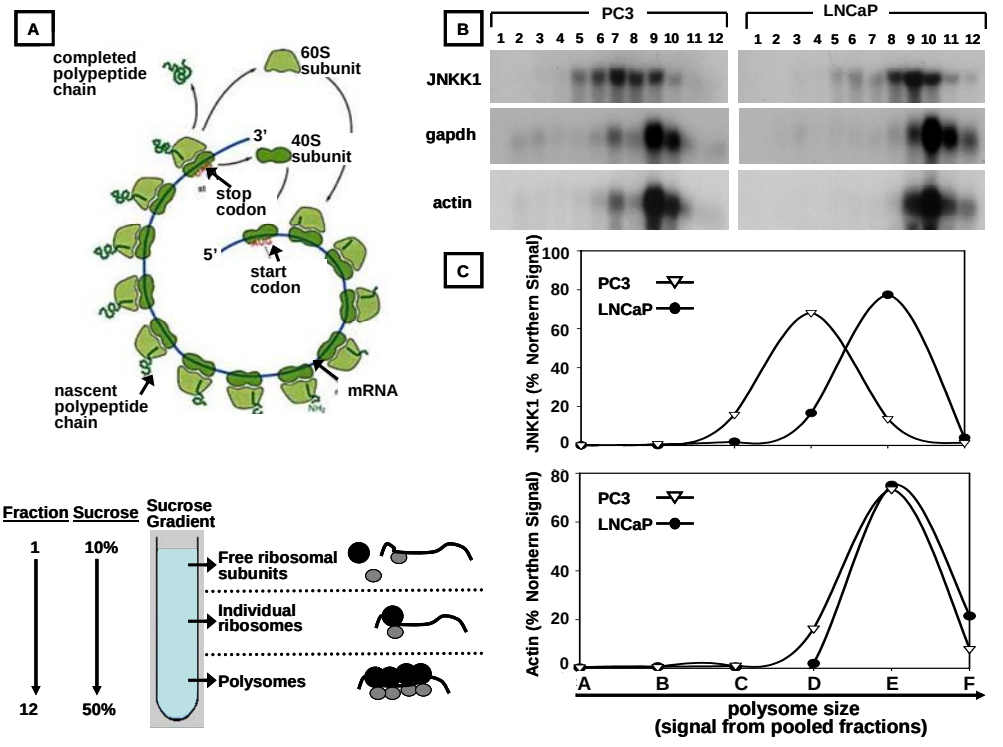


Figure. 2. MKK4 mRNA is preferentially associated with higher molecular weight polysomes in LNCap and C4-2 cells. A) Summary of polysome formation and analysis. *Upper Panel* Multiple ribosomes can bind to and translate a message. Ribosome cycling can be facilitated by interactions between 5' initiation factors and 3' binding proteins. *Lower Panel* Schematic of polysome analysis, a well-established method for the evaluation of translational engagement of a particular mRNA. B) Northern blot of MKK4 mRNA in individual fractions from a polysome gradient. C) Graphical representation of location of percent MKK4 mRNA signal vs. polysome size. The six data points shown on the X axis represent combined fractions from the gradient (i.e. 1= fractions 1 and 2, 2 = fractions 2 and 3) shown in the Northern (B)

Experiment D: Is MKK4 protein turnover higher in low-expressing PC3 cells compared to control epithelial cells? To determine the relative stability of MKK4 protein in cells expressing different MKK4 levels, we treated low- and high-expressing lines with cycloheximide (CHX) to block ribosomal function. We then assessed MKK4 protein levels by immunoblotting (IB) to monitor the rate of MKK4 protein loss, which serves as a measure of its relative half-life. Representative data from PC3 and LNCaP cells are shown in Figure 3. MKK4 levels were unaltered even after 24 hr of CHX treatment in both LNCaP (high MKK4-expressing cells) and PC3 (low MKK4-expressing cells), further indicating that the protein is quite stable (Figure 3, Panel A). C-myc was used as a control for this assay, as it has a short half-life. These data suggest that MKK4 protein

is highly stable and that protein degradation is unlikely to contribute to MKK4 down-regulation in low-expressing cell lines.

Experiment C: Are elements present in the MKK3 5' and 3' untranslated regions responsible for MKK4 downregulation? As an additional measure of the stability of MKK4 and the presence of regulatory sequences in the 5' and 3' UTRs, we ectopically expressed MKK4 in PC3 cells, which have low endogenous levels of MKK4. Stable cell lines expressing a construct containing the MKK4 coding region with a 5' HA tag (without endogenous UTR sequences), were subjected to a selection process, and clonal cell lines with the vector integrated in their genome were screened for expression of the HA epitope. HA-MKK4 expression was confirmed after 10 passages (Figure. 3, Panel B). Clonal lines using this construct have also been derived from the rat prostate cancer line AT6.1 and the human ovarian cancer line SKOV3ip.1. Thus, MKK4 protein is not inherently unstable in at least three independent cell lines.

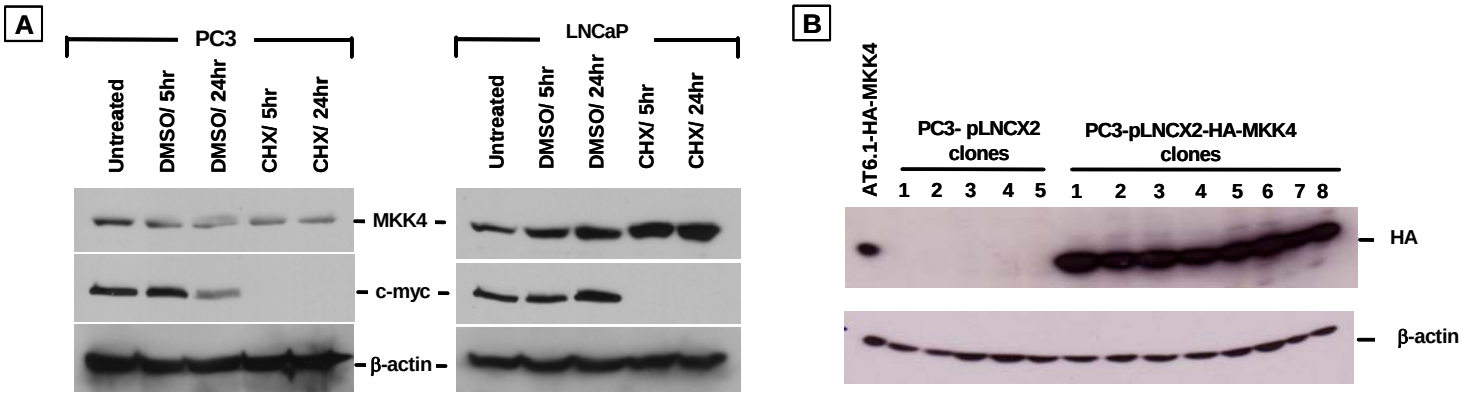
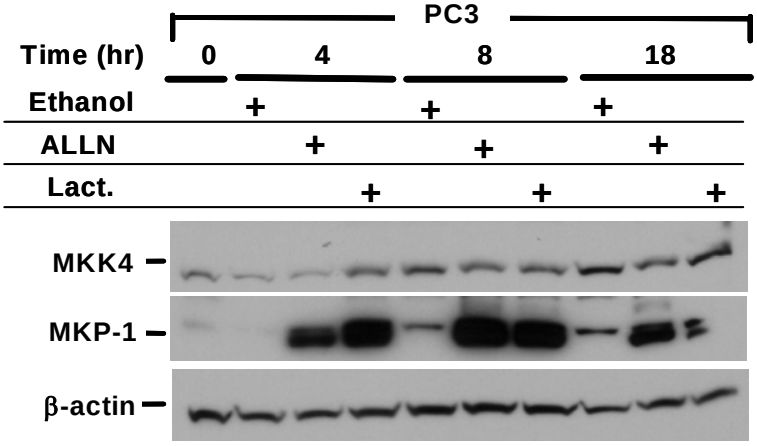


Figure 3. MKK4 protein is stable. (A) *MKK4 stability in low- (PC3) and high- (LNCaP) expressing cell lines.* Cells were treated with cycloheximide [CHX, 200 µg/ml prepared in DMSO (Sigma)] to inhibit ribosome function. Protein lysates were collected and 100 µg of each sample was used for immunoblotting. C-myc was used as a positive control for CHX function, as it has a half-life of 30-90 min. Actin served as a loading control. (B) *Expression of MKK4 in the absence of 5' and 3' UTR sequences.* Low-expressing cells (PC3) were transfected with an expression construct containing the MKK4 coding region tagged with an N-terminal HA. Ectopically expressed HA-tagged MKK4 was detected by IB for HA in stable clonal PC3 cell

Experiment E: Is MKK4 protein subject to ubiquitin-proteosome mediated degradation? The ubiquitin-proteasome degradation pathway is used by cells to degrade proteins that are no longer needed to recycle their amino acid components to form new proteins and it represents a key regulatory step in protein expression. To test the potential role of this pathway in the regulation of MKK4 protein levels, cell lines which express low levels of MKK4 were treated with the proteasome inhibitors ALLN (N-Acetyl-Leu-Leu-Norlea-al) (inhibitor of proteasomes and cysteine proteases), Lactacystin (specific inhibitor of proteasomes), or vehicle control (ethanol). Cells were treated for specific time intervals and lysates collected were examined for MKK4 expression (Figure 4). Treatment with proteasome inhibitors did not result in accumulation of MKK4 protein, demonstrating that proteasome-mediated degradation is unlikely to contribute to the regulation of MKK4

Figure 4. MKK4 protein is not subject to proteasome-mediated degradation. PC3 cells, which have low endogenous levels of MKK4 protein, were treated with ALLN (10µM) or Lactacystin (10µM) proteasome inhibitors, or ethanol vehicle control. Total protein lysates were collected and 100 µg of protein was subjected to SDS-PAGE followed by immunoblotting with anti-MKK4 antibody (). The membrane was stripped and reprobed for MKP-1, a protein known to be degraded by the ubiquitin-proteasome pathway, as a positive control. The membrane was stripped and reprobed for actin as a loading control.



abundance and suggesting that MKK4 is a stable protein. The levels of the proteasome-regulated protein MKP-1 were assessed as an internal control. As anticipated, MKP-1 protein accumulated following treatment with inhibitors.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated a discordance between MKK4 protein and message levels in a panel of human prostate cancer cell lines.
- Showed that decreased protein levels are not due to decreased mRNA.
- Showed that MKK4 protein is stable and not subject to proteosomal degradation.
- Found that MKK4 mRNA is primarily nuclear and that the cellular distribution of message does not account for differences in protein levels in high- and low- MKK4-expressing cell lines.
- Found evidence for the altered translational engagements (i.e. ribosomal localization) in low- and high-MKK4-expressing cells.
- Taken together these data suggest that MKK4 protein levels are regulated at least in part at the translational level.

REPORTABLE OUTCOMES: Reportable outcomes that have resulted from this research to include:

DISSERTATION:

Victoria L. Robinson: Post-transcriptional regulation of MKK4: A stress signaling kinase implicated in the regulation of metastatic growth." The University of Chicago Biological Sciences Division, June 2006.

ABSTRACTS:

Robinson, V.L., Gorospe, M., and Rinker-Schaeffer, C.W. "Post-transcriptional regulation of MKK4- A stress signaling kinase implicated in the regulation of metastatic growth." The AACR Edward A. Smuckler Memorial Educational Workshop on The Pathobiology of Cancer. Snowmass, CO, July 2005.

Robinson, V.L. and Rinker-Schaeffer, C.W. "The metastasis suppressor MKK4 is not down-regulated by a protein turnover mechanism." Keystone Symposia: The Role of Microenvironment in Tumor Induction and Progression, Banff, Canada, February, 2005.

MANUSCRIPT (in review):

Robinson, V.L., Shalhav, O., Otto, K., Kawai, T., Gorospe, M., Rinker-Schaeffer, C.W. Translational Regulation of MAP Kinase Kinase 4 (MKK4) Expression. *In Review at J. Biological Chemistry*.

CONCLUSION: There is increasing recognition that production of certain cellular proteins is temporally and spatially regulated at the level of protein translation. This mechanism of control endows cells with the ability to rapidly fine-tune levels of specific proteins in response to extracellular signals. In metazoans, the translational efficiencies of mRNAs vary over a 100-fold range. Translational efficiency is determined by sequence elements within the mRNA and proteins that interact with these sequences. Structural features and regulatory sequences within the mRNA which influence translational efficiency include: the 5' cap structure and 3'-poly (A) tail which modulate the efficiency of translational initiation; internal ribosome entry sites (IRESs), which mediate cap-independent translation initiation; upstream open reading frames (uORFs), which normally reduce translation from the main ORF; secondary or tertiary RNA structures, such as hairpins, which commonly block initiation but can also be part of IRES elements and therefore promote cap-independent translation; and sites within untranslated

regions (UTRs) for RNA binding proteins (RBPs) which modulate efficiency of mRNA translation. At this time there is no information about such structural elements in MKK4's UTRs.

Our data support a model in which MKK4 protein levels are controlled by recruitment of the MKK4 mRNA to polysomes. A mature mRNA contains the protein coding region flanked by 5' and 3' UTRs. These regions often contain regulatory regions that are recognition sites for specific RNA-binding proteins (RBPs) and/or microRNAs (miRNA). These interactions modulate the expression of the corresponding protein by influencing factors such as mRNA translational initiation. The observation that we can stably express MKK4 protein using constructs that contain only the MKK4 coding region, but lack the 5' and 3' UTRs in cells with low endogenous protein expression, prompted the hypothesis that the untranslated regions of the *endogenous* MKK4 mRNA contains regulatory elements serving as sites mRNA/RBPs interactions contributing MKK4 translation. Using heterologous reporter constructs, ongoing efforts are aimed at mapping the specific RNA region(s), particularly within the 3' and 5' UTRs, involved in promoting or repressing the translation of the MKK4 mRNA.

The translational regulation of MKK4 protein expression could be regulated by a variety of trans-acting factors. At this time our data only show that MKK4 mRNA is associated with larger polysomes in high-expressing (e.g. LNCaP and C4-2) cells as compared to low-expressing (e.g. PC3 and DuPro) cells. The translation of MKK4 mRNA could either be promoted in high-expressers or repressed in low-expressers. Our current working model is that interactions with a trans-acting factor repress translation of MKK4 mRNA in low-expressing cell lines. Two logical candidate trans-factor types, RBPs and MicroRNAs (miRNAs), can perform this putative translational repression mechanism. RBPs can associate co-transcriptionally with the nascent mRNA or bind at a subsequent time and participate in a variety of post-transcriptional processes, including 5' capping, 3' polyadenylation, recruitment to the ribosome, and modulation of translational efficiency. Some of the RBPs under consideration are proteins that associate with U- or AU-rich RNA elements, which are present throughout the MKK4 mRNA. These RBPs include two translational repressors, the T-cell-restricted intracellular antigen-1 (TIA-1) and the TIA-1-related protein TIAR, as well as HuR, a protein that has been shown to promote (2 refs.) or inhibit (2 refs.) translation in different experimental systems. In addition, to the more traditional RBPs, it is now recognized that miRNAs may silence gene expression in the cytoplasm by at least two different mechanisms. Perfectly complementary interactions between the miRNA and the target mRNA appear to result in mRNA degradation while mismatched interactions favor a repression of translation. Several miRNAs putatively targeting the MKK4 mRNA have been identified using computational methods. Their expression in our cell systems, as well as their influence upon MKK4 translation also awaits direct investigation.

We are in the midst of a revolution in the way we view metastasis and the molecular determinants of metastatic growth. Study of metastasis suppressor proteins is revealing new facets of the molecular regulation of metastases. Since metastasis suppressor genes are infrequently mutated, it has been postulated that therapies based on their "re-expression" may prove useful in controlling metastatic growth. The majority of studies addressing this issue have examined the transcriptional regulation of metastasis suppressor genes. To our knowledge, we are the first to examine the translational regulation of a metastasis suppressor. There is strong precedent for the translational control of proteins involved in regulation of key aspects of tumor initiation and progression. Furthermore, therapies targeting translational control are in clinical trials. As we expand our understanding of MKK4 in metastatic growth, it will be essential to elucidate the mechanisms governing MKK4 expression in order to identify novel targets of therapeutic intervention.

REFERENCES: All materials presented in this report are described in detail in the manuscript provided in the Appendix. This manuscript also contains all appropriate citations.

APPENDICES: The manuscript by Robinson *et al.* is provided in the following Appendix.

TRANSLATIONAL REGULATION OF MAP KINASE KINASE 4 (MKK4) EXPRESSION

Victoria L. Robinson‡, Ore Shalhav‡, Kristen Otto‡, Tomoko Kawai§, Myriam Gorospe§, and Carrie W. Rinker-Schaeffer‡¶

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Running Title: Translational Regulation of MKK4

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Mitogen-activated protein kinase kinase 4 (MKK4) is a dual-specificity kinase with a critical role in regulating the activity of the c-Jun-N-terminal kinase (JNK) and p38 kinases. We identified a novel biological function for MKK4 in the regulation of growth of ovarian and prostate cancer metastases. Previous clinical correlative studies showed that MKK4 protein levels were reduced in high-grade prostate cancer and prostate and ovarian cancer metastases as compared to normal tissue controls. These findings prompted the investigation of mechanism(s) responsible for down-regulation of MKK4 in a panel of cancer cell lines. Initial studies found that low levels of MKK4 protein did not correlate with either exon deletion or decreased levels of MKK4 mRNA, suggesting that MKK4 protein levels were regulated by either reduced translation or reduced protein stability. The endogenous MKK4 protein was very stable and did not appear to be subject to altered proteolysis. Instead, MKK4 biosynthesis appeared may be regulated by altered translation. In support of this assertion, we found that the cytosolic MKK4 mRNA was shifted towards active polysomes in cells with higher levels of MKK4 protein, suggesting that MKK4 mRNA was translated more efficiently in these cells. This study supports a novel mechanism for the regulation of MKK4 protein levels. Further, these findings have potential therapeutic implications for modulating the expression of an important signaling kinase involved in the regulation of metastatic growth.

MKK4 (also known as JNKK1, MEK4, and SEK1) is a widely expressed dual-specificity mitogen-activated protein kinase kinase (MAP2K) and a critical mediator of Stress- Activated Protein Kinase (SAPK) signaling (1-3). In response to cytokines or damaging stimuli such as pH changes and hypoxia, activated MKK4 can phosphorylate either the JNK or p38 MAPKs. Depending upon the cells' environment and the specific signal, MKK4 activation and the subsequent activation of JNK and/or p38 MAP kinases can lead to a variety of biological responses, including cell differentiation, growth, and death (4-6). Homozygous knockout of MKK4 in mice is embryonic lethal (7-9), and MKK4-null cells have deficiencies in JNK- and p38-dependent signaling (9).

We previously identified a novel function for MKK4 in the regulation of metastasis formation in both prostate and ovarian cancer models. Clinical correlative studies suggested the involvement of MKK4 down-regulation in the acquisition of metastatic ability of certain cancers. Specifically, immunohistochemical studies showed high levels of MKK4 protein in the epithelial, but not the stromal compartment, of normal prostatic and ovarian tissues. MKK4 protein was found to be decreased in advanced prostate cancers and ovarian cancer metastases, and the gene is infrequently mutated in cancer metastases (10,11). Similarly, in prostate and ovarian cancer cell lines, expression of MKK4 was lost or down-regulated in many of the cell lines evaluated (10,11). Immunohistochemical analysis of pancreatic cancers revealed that decreases in MKK4 protein expression correlated with poor prognosis (12). Additionally, in a study of breast cancer metastases in the brain, Stark *et al.* reported finding decreased MKK4 mRNA by RT-PCR and corresponding decreases in protein levels (13). These studies demonstrate that the MKK4 protein is frequently down-regulated in cancers and support a role for dysregulation of its signaling cascade in clinical disease.

The mechanism(s) of MKK4 inactivation in cancer is not well understood. Initial studies found that 15% of breast cancer cell lines harbored MKK4 genetic mutations (14,15), but additional studies suggest that the percentage of mutation in breast cancer cell lines may be much lower, comparable to the 2-5% of mutations found in all cancer lines tested to date (16). Chae *et al.* reported finding no MKK4 mutations in gastric carcinoma cell lines, as well as no decrease in MKK4 mRNA or protein in primary gastric carcinomas (17).

Taken together, findings from clinical and experimental studies prompted the question, "How is MKK4 protein down-regulated in cancer cells?" Although MKK4's kinase activity has been studied extensively, there is a dearth of information regarding the mechanisms that control endogenous protein levels. We sought to determine the mechanism by which MKK4 protein levels are modulated using high- and low MKK4-expressing prostate and ovarian cancer cell lines as a model system.

To test the hypothesis that decreased wild-type MKK4 protein expression levels could result from post-transcriptional regulatory events, we studied the mechanisms controlling protein expression in high and low MKK4-expressing human cancer cell lines. Studies were conducted to determine whether the two cell types displayed differences in 1) the relative stability of MKK4 protein, and 2) the relative translation of the MKK4 mRNA. Our studies found that MKK4 was highly stable in all cell lines tested and displayed no differential sensitivity to protease inhibitors. The compartmentalization of the MKK4 mRNA was also unchanged when comparing high- and low-expressing cells, all showing a highly stable transcript with a predominantly nuclear localization. Interestingly, however, within the cytoplasmic MKK4 mRNA subset, there was a distinct increase in the association of the MKK4 mRNA with the translational machinery in high MKK4-expressing cells compared to low MKK4-expressing cells. These findings suggest that MKK4 expression levels are influenced by the translational engagement of the MKK4 mRNA.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions – PC3, LNCap, C4-2, DuPro, and DU145 human prostate carcinoma cell lines were cultured in RPMI (Mediatech) supplemented with 10% FCS (Atlanta Biologicals), 1% penicillin (100 units/ml)/ streptomycin (100 µg/ml) (BioWhittaker/ Cambrex). For PC3-HA-MKK4 clonal cell lines which stably express the HA-MKK4 protein, standard media was additionally supplemented with G418 (Cellgro). SKOV3ip.1, SKOV3, CaOV3, HeyA8 human ovarian carcinoma cells, IOSE-T transformed ovarian epithelial cells, and HeLa cervical carcinoma cells were cultured in DMEM with L-glutamine and high glucose (4.5 g/L) (Mediatech), supplemented with 5% FCS, 1% penicillin/streptomycin, 10 mM Sodium Pyruvate (Mediatech), 1x Non-Essential Amino Acids (Mediatech), 2x MEM Vitamin Solution (Mediatech). ASPC-1 cells were grown in DMEM supplemented with 20% FCS and 1% penicillin/ streptomycin (Mediatech). RetroPack PT67 Packaging cells (Clontech), were cultured in DMEM supplemented with 8% FCS, 1% penicillin/streptomycin, Sodium Pyruvate, Non-Essential Amino Acids, and MEM Vitamin Solution.

Derivation of PC3-HA-MKK4 clonal cell lines – Clonal cell lines expressing full-length HA-tagged MKK4 were derived as previously described (18-20). RetroPack PT67 retroviral packaging cell line (Clontech) were transiently transfected (Qiagen Effectene) per the manufacturer's instructions. Viral media was collected 48 hours after transfection and passed through a 0.2 µm filter. PC3 cells were incubated with filtered viral media and Polybrene (SpecialtyMedia, Phillipsburg, NJ) for 24 hours passaged in growth medium containing G418 for selection establishment of stable clones. Two weeks later individual colonies were selected using cloning rings, expanded, collected, and immunoblotted for expression of the HA epitope-tagged protein. Expression was again confirmed following 10 passages.

Polymerase Chain Reaction – Nested PCR was performed using standard methods (New England Biolabs). Products were run on 2% agarose/TBE gels containing 0.2 µg/ml ethidium bromide for visualization, and photographed. Primer sequences

used for amplification of the eleven exons of MKK4 were as previously described (14).

Protein Lysate Preparation and Immunoblotting – Monolayer cell cultures were washed in cold PBS and protein lysates prepared by addition of chilled RIPA buffer [1x PBS, 1% IGEPAL CA-630, 0.1% SDS, 0.5% Na Deoxycholate] with the addition of a complete protease inhibitor pellet (Roche), 100 ng/ml PMSF (Sigma), 30 µl/ml Aprotinin (Sigma), and 3 mM monovalent $NaVO_4$ followed by scraping to remove them from the dish. The RIPA buffer-cell mixture was passed through a 27-gauge needle, transferred into a sterile tube, and centrifuged at 14,000 rpm at 4° C for 10 minutes. Protein concentrations were were quantified using the Pierce BCA Protein Assay kit. For immunoblotting 20-100 µg of total protein was resolved by SDS-PAGE on 10% gels under standard conditions and subsequently transferred to a HyBond ECL nitrocellulose membrane (Amersham). Membranes were blocked at 4^{o}C for 2-16 hours in TBS-T plus 5% w/v Carnation Non-Fat Dry Milk, incubated for 1 hour in primary antibody diluted in blocking solution, washed 6 times for 5 minutes each in TBS-T, followed by secondary antibody and washes. The HRP-conjugated secondary antibody was detected using the Super Signal West Femto Maximum Sensitivity Chemilluminescence Substrate (Pierce) per the manufacturer's directions. Probed membranes were stripped using Pierce Restore Western Blot Stripping Buffer or 2% SDS, 62.5 mM Tris-Cl, pH 6.8, 100 mM Beta-mercaptoethanol. The antibodies and dilutions are as follows: HA.11 (Covance, 1:1,000), MKK4 (Santa Cruz, 1:5,000), c-myc (Cell Signaling, 1:500), MKP-1 (SantaCruz, 1:500), Tubulin (SantaCruz, 1:1,000); Lamin A/C (Upstate, 1:2,000), β-actin (Calbiochem, 1:10,000); Anti-Rabbit-HRP (Cell Signaling, 1:10,000), Anti-Mouse IgG-HRP (Sigma, 1:10,000), Anti-Mouse-IgM-HRP (Calbiochem,1:50,000). Densitometric analysis was performed utilizing Un-Scan-It software (Silk Scientific). MKK4 protein expression was normalized against actin protein expression from the corresponding lysates. The ratios LNCap/PC3 and C4-2/DuPro were calculated to yield fold increases of 23.81 and 16.15, respectively.

Northern Blotting – Total RNA was extracted with TRIzol reagent and chloroform as directed (Invitrogen), precipitated with isopropanol, washed in 70% EtOH, and resuspended in molecular-grade water (Cambrex). For poly (A)+ mRNA isolation, Oligotex oligo-dT bead technology was used (Qiagen). RNA samples containing 50 ng/µl ethidium bromide were resolved on 1.2% agarose/MOPS gels (Cambrex), imaged and photographed. Capillary transfer of RNA onto a Zeta-probe membrane (BioRad) was performed overnight in 10X SSC and subjected to UV crosslinking. Random primer labeling of probes with [γ-^{32}P] dCTP was performed using the Megaprime DNA Labeling System (Amersham). Unincorporated nucleotides were removed using Chroma-spin 100 columns (Clontech). Membranes were pre-hybridized for 30 minutes in Express-Hyb Solution (Clontech) and the radioactive probe subsequently hybridized overnight (both at 65°C). Membranes were washed in 2x SSC/ 0.05% SDS, 3 x 30 minutes, followed by 0.1x SSC/ 0.1% SDS, 2 x 40 minutes and exposed on film at -80° C for 30 minutes to 24 hours. cDNA probes were as follows: MKK4 is a 1-kb fragment of MKK4 cDNA isolated by BglI/ SphI digestion of pLNCX2-jnkk1 plasmid (Vander Griend et al or Lin et al), GAPDH is a 0.9-kb fragment of human GAPDH amplified by PCR (primer sequences: 5' GAGTCAACGGATTTGGTCGT 3' and 5' TGAGCTTGACAAAGTGGTCG 3', and β-actin was a 838-bp cDNA (Biochain).

Drug Treatments – Drug solutions and vehicle controls were prepared to final concentrations in the appropriate media and cells incubated for the indicated times. Concentrations were as follows: 200 µg/ml cycloheximide (Sigma), 10 µM ALLN (Sigma), 10 µM lactacystin (Sigma), ethanol (1 µl/ml), 10 µg/ml actinomycin D (Sigma), 2 µl/ml DMSO.

Polysome Isolation – Cells were incubated with cycloheximide (0.1 mg/ml) for 20 minutes, washed with PBS, lysed with Polysome Extraction Buffer (PEB) [0.3 M NaCl, 15 mM $MgCl_2$, 15 mM Tris-Cl pH 7.6, 1% Triton X-100, 0.1 mg/ml cycloheximide, 1 mg/ml heparin], incubated on ice for 10 minutes and centrifuged at 14,000 rpm for

10 minutes to remove nuclear and membrane material. The soluble protein concentration was measured as above in order to normalize the samples. Lysates (2 mg) were brought to a final volume of 1 ml with PEB and layered onto sucrose gradients. Sucrose gradients were prepared with 2.2 ml 10-50% sucrose layers in 0.3 M NaCl, 15 mM $MgCl_2$, 15 mM Tris-Cl pH 7.6, 0.1 mg/ml cycloheximide, 1 mg/ml heparin, and allowed to settle overnight at 4ºC. Gradients were centrifuged in a Beckman Ultracentrifuge SW-41 rotor at 39,000 rpm for 90 minutes. Fractions (1 ml) were collected, vortexed with 1.5 ml of Guanidine HCl, and RNA was precipitated with ethanol. Northern blotting was performed as above.

Nuclear Fractionation – To prepare fractions, the Biovision Nuclear/Cytosol Fractionation Kit was used according to manufacturer's instructions with the following addition: the RNase inhibitor Superasin (Ambion) was added to buffers for a final concentration of 0.1 U/ul to preserve RNA integrity. $1x10^6$ cells were fractionated ($5x10^5$ in two separate tubes), nuclear and cytoplasmic fractions pooled, and then divided in half for RNA and protein preparation as described above. Northern and immunoblotting analysis was performed as described above.

RESULTS

We previously observed low endogenous MKK4 levels in many human prostate and ovarian cancer cell lines (10,11). As shown in Fig. 1A, the human prostate cancer (CaP) cell lines DU145, PC3, and DuPro have low to undetectable MKK4 protein levels under standard assay conditions (20 μg). Immunoblotting with 100 μg of total protein facilitated detection of MKK4 in all cell lines examined, and revealed that low MKK4-expressing cell lines have approximately 20-fold less MKK4, as compared to the high MKK4-expressing lines LNCaP and C4-2 (Fig. 1A, left). Cell lines of ovarian and cervical origin were examined to extend this initial finding. Interestingly, all of these cell lines had low endogenous levels of MKK4 protein, which could only be detected when 100 μg of total protein was examined. To rule out the possibility that decreased levels of MKK4 protein were due to genomic deletion (s), cell lines were screened for the presence of MKK4 exons A-K. Representative data are shown in Fig. 1 B. Total human genomic DNA was used as a positive control while DNA prepared from the human pancreatic cancer cell line ASPC-1, which harbors homozygous deletion of exons B and C of MKK4, was used as a negative control (14). All exons of the MKK4 gene were present in each of the four prostate cancer cell lines (PC3, LNCaP, C4-2, and DU145) (Fig. 1B). Similarly, three ovarian cancer cell lines (HeyA8, SKOV3, SKOV3ip.1) were positive for all exons (data not shown). To test the possibility that decreased MKK4 levels were due to decreased levels of MKK4 RNA, the relative amount of MKK4 mRNA present in low- and high-expressing cells lines was assessed. Northern analysis showed that both high- and low-expressing prostate cancer cell lines had comparable levels of MKK4 mRNA (Fig. 1C, left panels). Similar results were obtained in ovarian cancer cell lines, as robust expression of MKK4 mRNA was detected in HeyA8, SKOV3, SKOV3ip.1, CaOv3 (Fig.1C, right panels), although expression is lower than prostate cancer cells. Moreover, HeLa cervical cancer cells and IOSE-T immortalized ovarian epithelial cells have similar levels of MKK4 mRNA. Actinomycin D treatment of PC3, LNCaP, and SKOV3ip.1 cells revealed that MKK4 mRNA stability is similar between cells lines (data not shown). The discrepancy between the relative levels of MKK4 protein and mRNA observed in many of these cell lines led us to hypothesize that the steady state level of MKK4 protein is regulated by a post-transcriptional mechanism.

To begin to test this hypothesis, we first considered the possibility that low levels of MKK4 protein were due to differential MKK4 protein turnover. A prominent protein elimination system is the ubiquitin-proteasome degradation pathway, used by cells to regulate protein levels and to degrade proteins in order to recycle their amino acid components to form new proteins (21). PC3, LNCaP, HeLa, and SKOV3ip.1 cells were treated with the proteasome inhibitors ALLN (N-Acetyl-Leu-Leu-Norlea-al) (inhibitor of the proteasome and cysteine proteases), lactacystin (specific inhibitor of proteasome), or vehicle control, with cell lysates collected at specific time intervals, followed by immunoblotting for MKK4.

Representative data from PC3 and SKOV3ip.1 cells are shown in Fig. 2. Treatment with proteasome inhibitors did not result in the accumulation of MKK4 protein, indicating that proteasome-mediated degradation does not contribute significantly to the regulation of MKK4 abundance, and suggests that MKK4 is a stable protein. The levels of the proteasome-regulated protein MKP-1 were assessed as an internal control. As anticipated, MKP-1 protein accumulated following treatment with inhibitors (22). β-actin served as a loading control.

To determine the relative stability of MKK4 protein in cells expressing different MKK4 levels, we treated PC3, LNCaP, SKOV3ip.1, and HeLa cells with cycloheximide (CHX) to block ribosomal function. We then assessed MKK4 protein levels by immunoblotting to monitor the rate of MKK4 protein loss, which serves as a measure of its relative half-life. Fig. 3 illustrates the finding that MKK4 levels were unaltered even after 24 hours of CHX treatment in both LNCaP (high MKK4-expressing cells) and PC3 (low MKK4-expressing cells), further indicating that the protein is quite stable. C-myc was used as a control for this assay, as it has a short half-life (23). CHX treatment of SKOV3ip.1 and HeLa cells yielded similar results (data not shown). These data suggest that MKK4 protein is highly stable and that protein degradation is unlikely to contribute to MKK4 down-regulation in low-expressing cell lines.

As a third measure of the stability of MKK4, we ectopically expressed MKK4 in PC3 cells. Stable cell lines expressing a construct containing the MKK4 coding region with a 5' HA tag [without endogenous untranslated (UTR) sequences]were subjected to a selection process, and clonal cell lines harboring genome-integrated vector were screened for expression of the HA epitope. HA-MKK4 expression was confirmed after 10 passages (Fig. 3). Our laboratory previously determined that HA-tagged MKK4 can be stably expressed at high levels in two cell lines-AT6.1 rat prostate cancer cells (19), and SKOV3ip.1 human ovarian cancer cells (11,20). Thus, exogenously expressed full-length MKK4 protein is stable in cell lines of different origins (including two human cell lines with low MKK4 protein), suggesting that MKK4 is not inherently unstable.

Next, we examined the possibility that decreased protein production is due to a mechanism involving RNA transport and/ or translation. A critical, though often overlooked, step in gene regulation is the transport of mRNA from the nucleus to the cytoplasm, where it is translated into protein (24). The bulk of cellular mRNAs appears to be exported at constant rates, but mRNA transport has been shown to critically dictate the expression patterns for some genes (25). To test the possibility that MKK4 mRNA is inefficiently transported from the nucleus in low MKK4-expressing cells, PC3 and LNCaP cells were fractionated into cytosolic and nuclear components. Northern blotting of cellular fractions demonstrated that MKK4 mRNA is primarily nuclear in both cell lines (Fig. 4), and was not preferentially elevated in the cytoplasm of LNCaP cells. Protein was also isolated from fractions and cytoplamic and nuclear markers (α-Tubulin and Lamin A/C, respectively) were immunoblotted to confirm the complete and specific preparation of cytoplasmic and nuclear lysates.

Having systematically excluded the involvement of key gene regulatory mechanisms in determining MKK4 abundance, we asked whether the relative translation rate of MKK4 mRNA may underlie the observed differences in MKK4 protein levels. To this end, we studied the association of the MKK4 mRNA with the translational machinery in cells expressing different levels of MKK4. Although there are many levels of control within the process of translation, translational initiation is often the rate-limiting step (26). The association of a given mRNA with high-molecular weight polysomes (complexes of multiple ribosomes translating a single mRNA) reveals the degree to which that mRNA has completed initiation and is engaged in active translation. Consequently, analysis of polysome-associated mRNA provides a measure of the synthesis rate of the corresponding protein (27). To ascertain the translational status of the MKK4 mRNA, we investigated its relative association with the translational apparatus. Cytoplasmic lysates (containing ribosomal subunits, individual ribosomes, and polysomes of increasing molecular weight) were size-fractionated using sucrose-density gradients. Following the extraction of total RNA, the relative levels of the MKK4, and GAPDH and β-Actin

housekeeping gene mRNAs in each fraction were examined by northern blot analysis. A comparison of lysates from low MKK4-expressing (PC3 and DuPro) and high MKK4-expressing (LNCaP and C4-2) cells revealed that the MKK4 mRNA in LNCaP and C-42 cells was preferentially associated with polysomes of higher molecular weight, indicating a greater engagement of MKK4 in translation in these cells (Fig. 5). Together, these observations point to the critical involvement of translational regulation as a key process governing the levels of MKK4 protein.

DISCUSSION

There is increasing recognition that production of a variety of cellular proteins is temporally and spatially regulated at the level of protein translation. This mechanism of control endows cells with the ability to rapidly fine-tune levels of individual proteins in response to situation-specific signals. It is now believed that only a modest correlation exists between mRNA and protein levels in eukaryotes, and translational efficiencies of mRNAs vary widely.

Translational efficiency is determined by sequence ('*cis*') elements within the mRNA and by '*trans*' factors that interact with these sequences. In addition to ubiquitous constituents of virtually all mammalian mRNAs (the 5' cap structure and 3'-poly (A) tail), a number of structural features and regulatory sequences within the mRNA can influence translational efficiency. Theses include: internal ribosome entry sites (IRESs), which mediate cap-independent translation initiation; upstream open reading frames (uORFs), which normally reduce translation from the main ORF; secondary or tertiary RNA structures, such as hairpins; and regulatory sites within untranslated regions (UTRs) (28). At this time, there is no information regarding structural elements within the MKK4 mRNA.

Our data support a model in which MKK4 protein levels are controlled by recruitment of the MKK4 mRNA to polysomes. The MKK4 mRNA UTRs may contain sequences responsible for regulating translation of the transcript, through interactions with regulatory molecules, such as RBPs and micro RNAs (miRNA). Normal cells may require a certain level of RBP(s) and/or miRNA(s) to maintain appropriate translation of MKK4 mRNA, and thus wild-type protein levels. If the levels or function of positive regulatory molecules are decreased, less mRNA is translated. Alternatively, cancer cells with less efficient translation could have upregulated an RBP or miRNA that binds to the UTR to prevent translation. In other words, there could be a mechanism required for maintaining MKK4 protein levels in normal cells, which is altered in low MKK4-expressing cancer cells, or there could be a cancer-specific mechanism that is not involved in the normal regulation of MKK4 translation.

The observation that we can stably express HA- tagged MKK4 protein in cells with low MKK4 levels using constructs that contain the MKK4 coding region, but lack the 5' and 3' UTRs, suggests that the UTRs of the *endogenous* MKK4 mRNA contain regulatory elements that contribute to translation. Using heterologous reporter constructs may help identify the specific regulatory regions in the MKK4 mRNA involved in controlling its translation.

An additional goal is to identify the translation-regulatory molecules that function by interacting with MKK4 mRNA. RBPs are a large group of proteins, and while many of these are required for the normal biogenesis of mRNAs, others directly regulate the amount of protein synthesized from a given transcript. Two possible mechanisms could explain RBP involvement in controlling MKK4 protein expression. In normal cells (and high MKK4-expressing cancer cells) an RBP could bind to the MKK4 mRNA and assist in promoting its translation. Alternatively, an RBP could bind to the transcript and function by repressing its translation. There are several candidate RBPs that regulate translation and have potential roles in cancer. HuR is a member of the ELAV family of RBPs (29), and may contribute to malignancy by controlling the expression of specific mRNAs (30). T-cell-restricted intracellular antigen-1 (TIA-1) and TIA-1-related protein (TIAR) are two translational repressors that have roles in development and stress responses (31). The influence of these and other RBPs on MKK4 translation is a current topic of interest.

MiRNAs function as post-transcriptional regulators of gene expression through a number of different mechanisms, including promoting RNA degradation, repressing translation initiation,

blocking protein elongation, and possibly additional mechanisms, such as engaging in complexes of sequestered mRNAs (28,32). In animals, miRNAs bind target mRNAs primarily in 3'UTRs, although not by conventional complementary base-pairing along their length, making it difficult to accurately predict the target mRNAs. In fact, only a few validated miRNA-target mRNA pairs have been described in mammalian systems (32). MKK4 is a putative target of over 30 miRNAs (33). Expression of these miRNAs, as well as examination of functional interactions with MKK4 mRNA awaits direct investigation.

We are in the midst of a revolution in the way we view metastasis and the molecular determinants of metastatic growth. Study of metastasis suppressor proteins is revealing new facets of the molecular regulation of metastases. The existence of a translational mechanism for control of MKK4 protein levels in prostate cancer cells, and possibly other cancer types, fits well into the overall view of metastasis suppressor regulation. Metastatic cells may need to quickly adapt to a new environment by altering levels of metastasis-related proteins. It may not be favorable for cancer cells to permanently alter metastasis genes (through mutation), as their function may be required at one or more steps of metastasis formation. Therapies based on "re-expression" of metastasis suppressors may be feasible if post-transcriptional mechanisms, such as translational suppression, control their expression. While several studies have demonstrated the contribution of transcriptional regulation in down-regulation of metastasis suppressors (34,35), this is the first investigation to reveal the translational regulation of a metastasis suppressor. There is strong precedent for the involvement of alterations in protein translation in tumor initiation and progression, on both the global and individual transcript level (36-40). As we expand our understanding of MKK4-mediated metastasis suppression, elucidating the specific mechanisms governing endogenous MKK4 expression will be essential for identifying novel targets for therapeutic intervention.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Low levels of MKK4 protein do not correlate with genomic deletions or low levels of MKK4 mRNA. (A) MKK4 protein expression in a panel of prostate, ovarian and cervical cancer cell lines. 20 μg or 100 μg of protein was immunoblotted for MKK4. Rat brain (5 μg) and ASPC-1 (20 μg or 100 μg) cell protein served as positive and negative controls for MKK4 expression, respectively. β-actin was used a loading control. Immunoblots are representative of data from six independent experiments. (B) Genomic DNA was isolated and PCR reactions were done with nested primers specific for each of the 11 exons (A-E) of the MKK4 gene. Human genomic DNA was used as a positive control for all reactions. Water and ASPC-1, a pancreatic cancer cell line harboring a homozygous deletion of MKK4 exons B and C, served as negative controls. Data are representative of two independent experiments. (C) MKK4 mRNA expression in cancer cell lines. Poly (A) mRNA was isolated from total RNA for northern blotting with a probe complementary to the MKK4 coding region. Rat brain and ASPC-1 cells served as positive and negative controls for MKK4 expression, respectively. Representative data from three independent experiments are shown.

Figure 2. Inhibition of the proteasome does not increase MKK4 protein levels. Cells were incubated with media supplemented with proteasome inhibitors (ALLN or lactacystin) or vehicle control (ethanol) for the indicated times. Protein lysates were prepared from HeLa and SKOV3ip.1 cells. 100 μg of total protein was immunoblotted for MKK4. Membranes were stripped and re-probed for MKP-1 as a control for inhibition of proteasome function, and subsequently stripped and re-probed for β-actin as a control for protein loading. Loss of MKP-1 expression in the lactacystin 18 hour time points may be due to hydrolysis of the covalent linkage between lactacystin and the proteasome (41). Representative data from at least three experiments are shown.

Figure 3. The MKK4 protein is highly stable. (A) Treatment of prostate cancer cells with cycloheximide does not result in degradation of MKK4 protein. PC3 cells and LNCaP cells were incubated with cycloheximide, or DMSO vehicle control, for 5 hours or 24 hours. Protein (100 μg) was immunoblotted for MKK4 (top panels). Membranes were stripped and re-probed with an antibody against c-myc as a control for inhibition of protein synthesis (middle panels). Membranes were stripped and re-probed for β-actin as a loading control (bottom panels). Representative data from at least three experiments are shown. (B) Stable expression of exogenous HA-MKK4 in clonal PC3 cell lines. PC3 cells were infected with pLNCX2-jnkk1 (MKK4) or pLNCX2-vector only retroviruses, and individual clonal cell lines were propagated. After 10 passages of tissue culture, cell protein lysates were immunoblotted for the HA tag to confirm expression of HA-MKK4, and β-actin as a control for protein loading. AT6.1-HA-MKK4 cells were used as a positive control for HA expression (19).

Figure 4. MKK4 mRNA is primarily nuclear. PC3 and LNCaP cells were fractionated into nuclear and cytoplasmic components, and RNA and protein samples were prepared. Total RNA (5 μg) was used for northern blot analysis to detect MKK4 and GAPDH mRNAs. Protein (20 μg) was used for immunoblotting for the nuclear markers Lamin A and C, and the cytoplasmic marker α-tubulin. Data are representative of three independent experiments.

Figure 5. Polysome analysis of cell lines expressing low (PC3, DuPro) and high (LNCaP, C4-2) levels of MKK4 protein. (A) Cytoplasmic ell lysates were separated in sucrose density gradients and fractions were collected. Fraction 1 corresponds to the lightest fraction, fraction 12 the heaviest. Total RNA was prepared from each fraction, separated by gel electrophoresis, and transferred to membranes for northern blotting with a probe spanning the MKK4 coding region. Membranes were stripped and re-probed to monitor the expression of GAPDH mRNA (encoding a housekeeping gene) in these fractions. Membranes were subsequently stripped and re-probed for expression of β-actin mRNA, encoding a second housekeeping gene. These two genes had strikingly similar mRNA patterns, supporting their use as a measure of actively translating polysome fractions. Representative data from three experiments are shown. (B) Graphic representative of polysome analysis. Pooled fractions are indicated as follows: A= fractions 1 and 2; B= fractions 2 and 3, C= fractions 4 and 5, etc.).

Figure 1

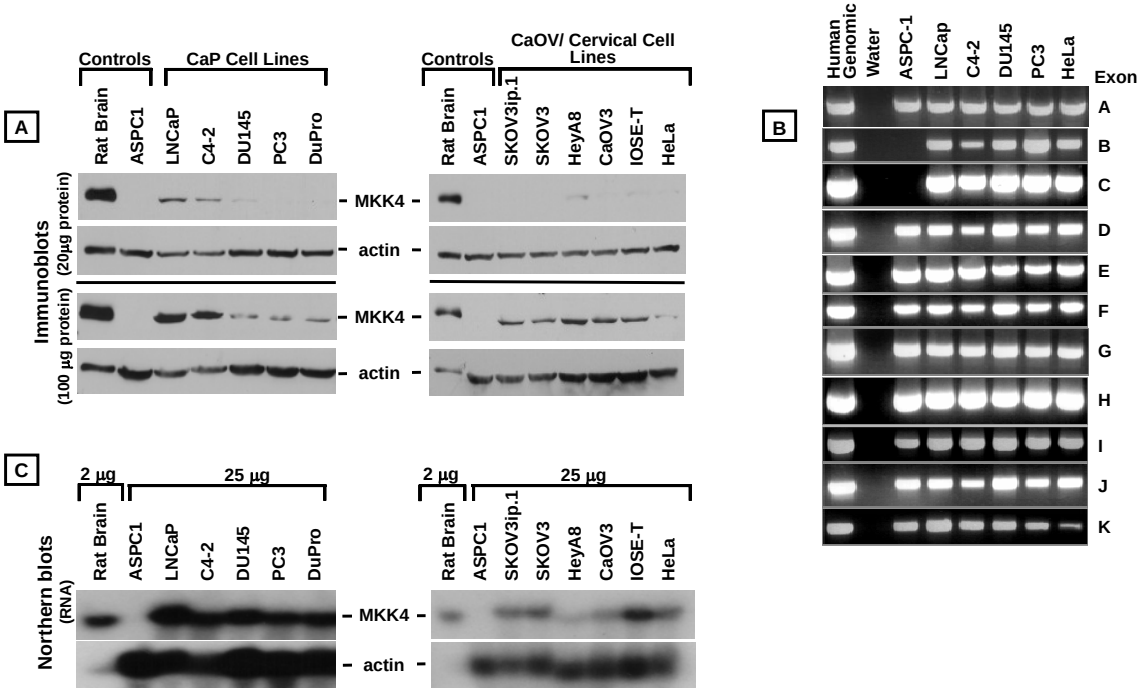

A
Controls
CaP Cell Lines
Controls
CaOV/ Cervical Cell Lines
Rat Brain
ASPC1
LNCaP
C4-2
DU145
PC3
DuPro
Rat Brain
ASPC1
SKOV3ip.1
SKOV3
HeyA8
CaOV3
IOSE-T
HeLa
Immunoblots
(20µg protein)
(100 µg protein)
MKK4
actin
MKK4
actin
B
Human Genomic
Water
ASPC-1
LNCap
C4-2
DU145
PC3
HeLa
Exon
A
B
C
D
E
F
G
H
I
J
K
C
2 µg
25 µg
2 µg
25 µg
Rat Brain
ASPC1
LNCaP
C4-2
DU145
PC3
DuPro
Rat Brain
ASPC1
SKOV3ip.1
SKOV3
HeyA8
CaOV3
IOSE-T
HeLa
Northern blots
(RNA)
MKK4
actin

Figure 2

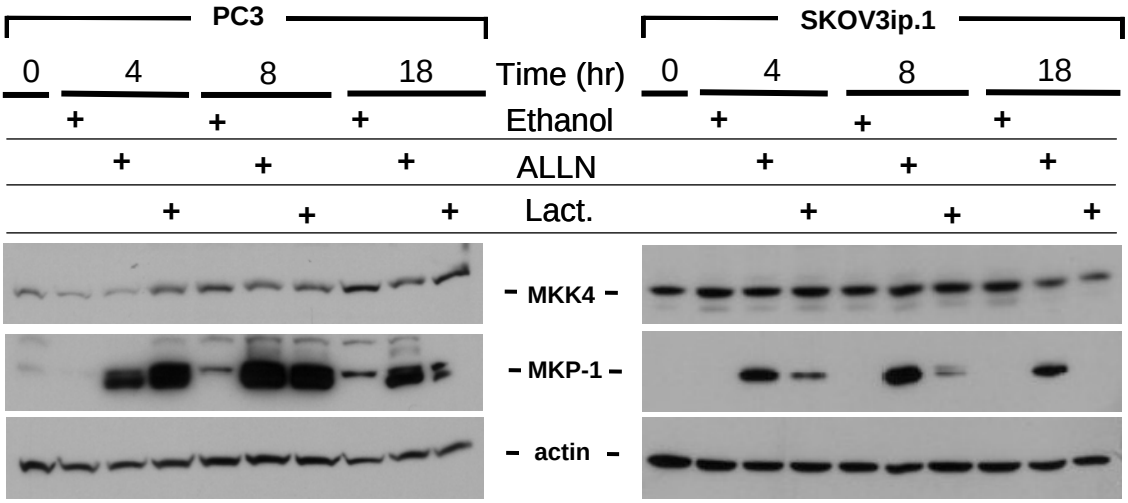

PC3
SKOV3ip.1
0
4
8
18
Time (hr)
Ethanol
ALLN
Lact.
MKK4
MKP-1
actin

Figure 3

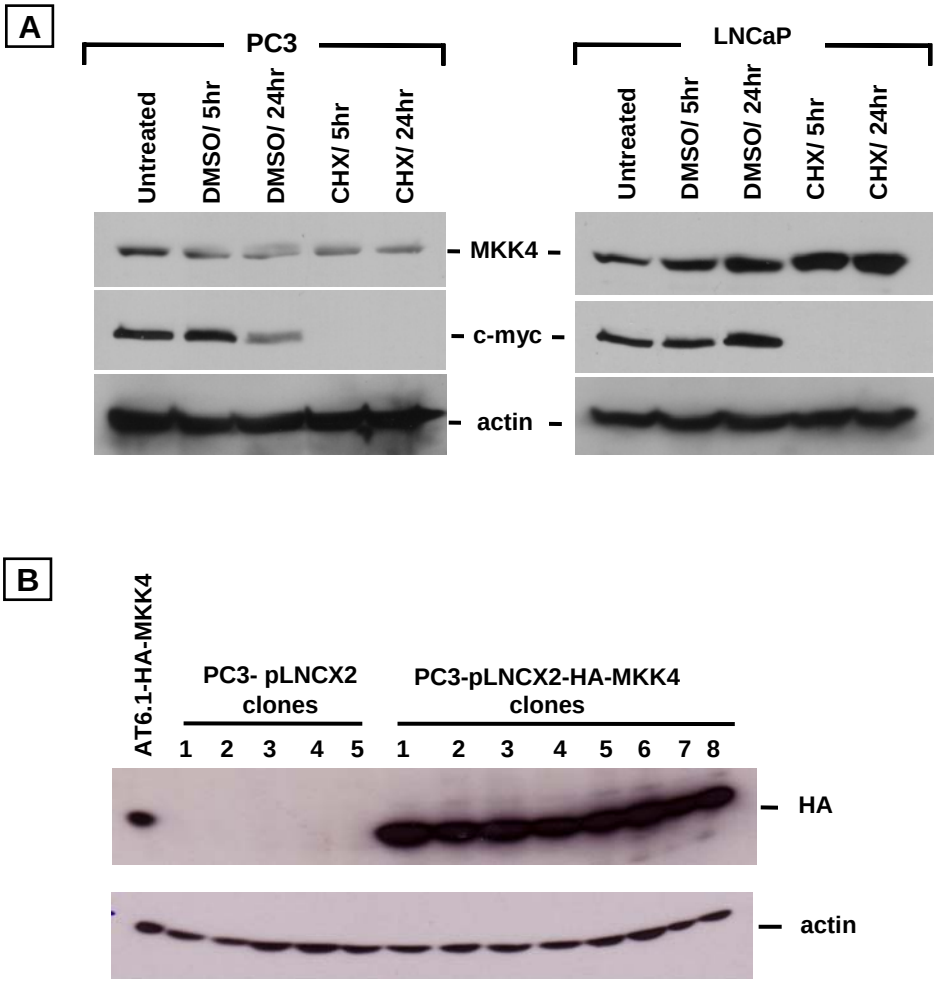

A
PC3
LNCaP
Untreated
DMSO/ 5hr
DMSO/ 24hr
CHX/ 5hr
CHX/ 24hr
Untreated
DMSO/ 5hr
DMSO/ 24hr
CHX/ 5hr
CHX/ 24hr
MKK4
c-myc
actin
B
AT6.1-HA-MKK4
PC3- pLNCX2 clones
PC3-pLNCX2-HA-MKK4 clones
1 2 3 4 5
1 2 3 4 5 6 7 8
HA
actin

Figure 4

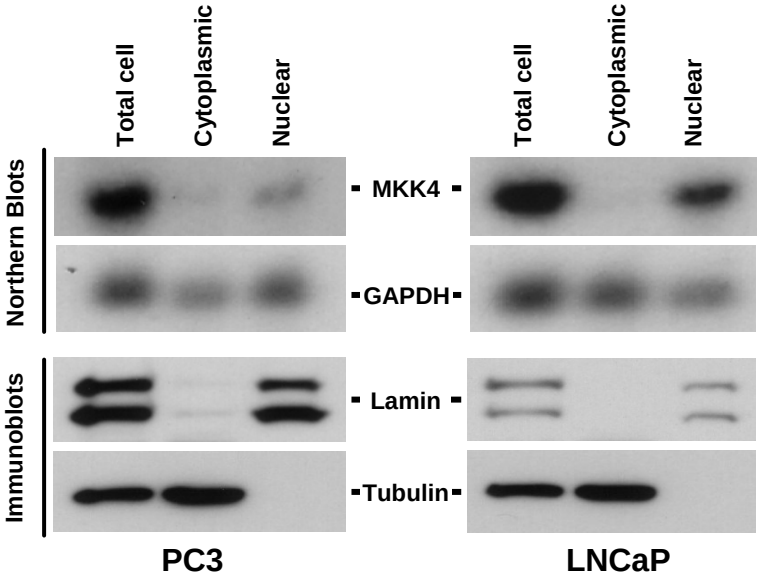
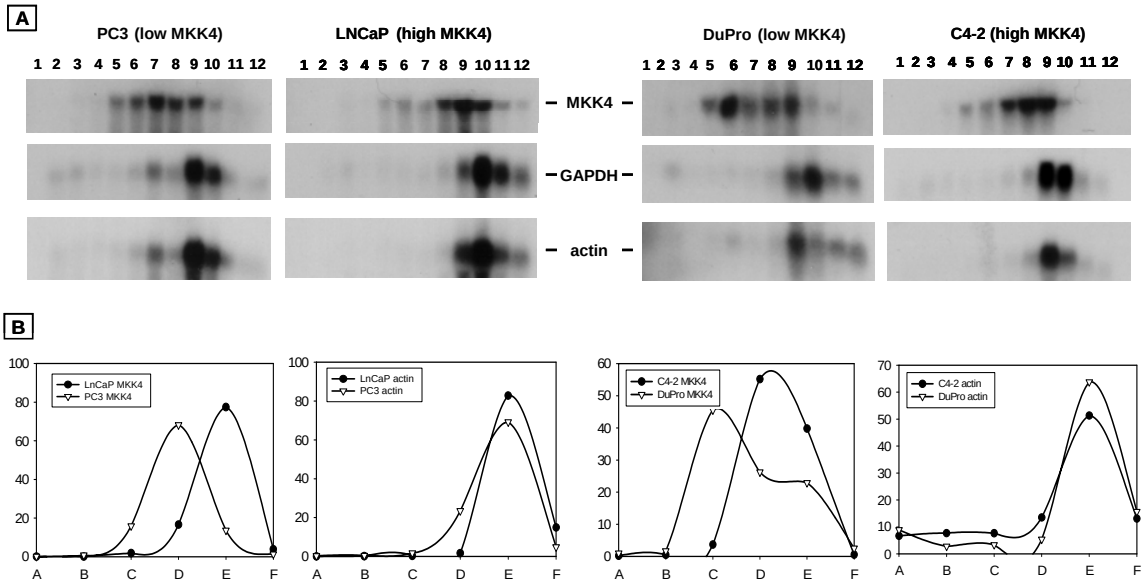

Total cell
Cytoplasmic
Nuclear
Total cell
Cytoplasmic
Nuclear
Northern Blots
Immunoblots
MKK4
GAPDH
Lamin
Tubulin
PC3
LNCaP

Figure 5


A
PC3 (low MKK4)
LNCaP (high MKK4)
DuPro (low MKK4)
C4-2 (high MKK4)
1 2 3 4 5 6 7 8 9 10 11 12
MKK4
GAPDH
actin
B
LnCaP MKK4
PC3 MKK4
LnCaP actin
PC3 actin
C4-2 MKK4
DuPro MKK4
C4-2 actin
DuPro actin
A B C D E F